



(19)

Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11)

EP 0 146 354 B2

(12)

## NEW EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention  
of the opposition decision:  
**24.10.2001 Bulletin 2001/43**

(51) Int Cl.<sup>7</sup>: **C12N 15/23, A61K 38/21,  
C12P 21/02, C07K 2/00,  
C07H 21/04**

(45) Mention of the grant of the patent:  
**13.03.1996 Bulletin 1996/11**

(21) Application number: **84308710.7**(22) Date of filing: **14.12.1984**(54) **Recombinant gamma interferons and pharmaceutical compositions containing them**

Rekombinante gamma-Interferonen und diese enthaltende Pharmazeutische Zusammensetzungen  
Gamma-interférons recombinants et composition pharmaceutique les contenant

(84) Designated Contracting States:  
**AT BE CH DE FR GB IT LI LU NL SE**

(30) Priority: **16.12.1983 US 562009  
27.02.1984 US 584217**

(43) Date of publication of application:  
**26.06.1985 Bulletin 1985/26**

(73) Proprietor: **GENENTECH, INC.**  
South San Francisco California 94080 (US)

(72) Inventors:  

- Gray, Patrick William  
San Francisco, CA 94127 (US)
- Rinderknecht, Ernst Heinrich  
San Carlos, CA 94070 (US)

(74) Representative: Armitage, Ian Michael et al  
MEWBURN ELLIS  
York House  
23 Kingsway  
London WC2B 6HP (GB)

(56) References cited:  

<b>EP-A- 0 077 670</b>	<b>EP-A- 0 083 777</b>
<b>WO-A-83/04053</b>	<b>GB-A- 2 107 718</b>

- EXP. MANIPULATION GENE EXPRESSION, 1983; R. DERYNCK et al., pp. 247-258
- JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 259, no. 11, 10 June 1984, American Society of Biological Chemists Inc., US; E. RINDERKNECHT et al., pp. 6790-6797
- NATURE, vol. 287, 1980; D. GOEDDEL et al., p. 411
- NATURE, vol. 290, 1981; D. GOEDDEL et al., p. 20
- NUCLEIC ACIDS RESEARCH, vol. 9, 1981; E. YERVERTON et al., p. 731

- ANNALS OF INTERNAL MEDICINE, vol. 93, 1980; GUTTERMAN et al., p. 399
- NUCLEIC ACIDS RESEARCH, vol. 8, 1980; D. GOEDDEL et al., p. 4057
- NATURE, vol. 295, 1982; P. GRAY et al., pp. 503-508
- NUCLEIC ACIDS RESEARCH, vol. 10, 1982; R. DERYNCK et al., p. 3605
- INTERFERON SCIENTIFIC MEMORANDA, August 1982; R. DERYNCK et al., Memo-I-A1193/2
- NATURE, vol. 287, 1980; D. GOEDDEL et al., pp. 411-416
- THE INTERFERON SYSTEM, Springer Verlag, New York, NY (US), 1979; pp. 13-26
- STEWART (ed.), "Interferon systems", Springer Verlag, New York, NY (US), 1979; p. 13
- NATURE, vol. 227, 1970; LAEMMLI, p. 680
- METHODS IN ENZYMOLOGY; GROSS et al., XI 238
- CELL, vol. 23, 1981; GLUZMAN, p. 175
- THE BIOLOGY OF THE INTERFERON SYSTEM, DeMaeyer and Schellekens (eds.), Elsevier Science Publ., 1983; pp. 119-128
- SCIENCE, vol. 222, 1983; BERMAN et al., p. 524
- MOLECULAR & CELLULAR BIOLOGY, vol. 3, 1983; SIMONSEN et al., p. 2250
- NATURE, vol. 292, 1981; NATHAN et al., pp. 842-844
- SCIENCE, vol. 215, 1981; YIP et al., pp. 411-413
- MICROBIOLOGY, 1980; VILCEK et al., pp. 204-207
- PROCEEDINGS OF THE NATL. ACADEMY OF SCIENCES USA, vol. 78, no. 3, 1981, Washington, DC (US); YIP et al., pp. 1601-1605
- NUCLEIC ACIDS RESEARCH, vol. 10, no. 8, 1982; DEVOS et al., pp. 2487-2501

- 3rd ANNUAL INTERNATL. CONGRESS FOR  
INTERFERON RESEARCH; CAPON et al., no.  
1982
- JOURNAL OF BIOLOGICAL CHEMISTRY, vol.  
259, no. 11, Juné 1984; RINDERKNECHT et al.,  
pp. 6790- 6797

**Description**

[0001] The present invention relates to the field of recombinant DNA technology, to means and methods utilizing such technology in the preparation of recombinant gamma interferons having enhanced stability, to their production and to the various products of such production and their uses.

[0002] This application is related to GB 2107718.

[0003] The publications and other materials used herein to illuminate the background of the invention and in particular cases to provide additional details respecting its practice are for convenience numerically referenced in the following text and respectively grouped in the appended bibliography.

[0004] Human interferons can be classified in three groups on the basis of different antigenicity and biological and biochemical properties. The first group comprises a family of leukocyte interferons which are normally produced mainly by constituent cells of human blood upon viral induction. These have been microbially produced and found to be biologically active (1,2,3). Their biological properties have prompted their use in the clinic as therapeutic agents for the treatment of viral infections and malignant conditions (4).

[0005] In the second group is human fibroblast interferon, normally produced by fibroblasts upon viral induction, which has likewise been microbially produced and found to exhibit a wide range of biological activities (5). Clinical trials also indicate its potential therapeutical value. The leukocyte and fibroblast interferons exhibit very clear similarities in their biological properties despite the fact that the degree of homology at the amino acid level is relatively low. Both groups of interferons contain from about 165 to about 166 amino acids and are acid stable proteins.

[0006] Human gamma interferon (also variously referred to as immune interferon,  $\gamma$ -interferon, IIF or IFN- $\gamma$ ) exhibits the antiviral and anti-proliferative properties characteristic of the interferons but, in contrast to leukocyte and fibroblast interferons, is pH 2 labile. Prior to the production of gamma interferons via recombinant DNA technology, it had been produced mainly upon mitogenic induction of lymphocytes. Human gamma interferon is clearly antigenically distinct from the leukocyte and fibroblast interferons. Gray, Goeddel and co-workers were the first to report expression of a recombinant gamma interferon (6), which has proven to exhibit the characteristic properties of human gamma interferon, i.e., anti-viral and anti-proliferative activity coupled with pH 2 lability. The recombinant gamma interferon of Gray and Goeddel, as produced in *E. coli*, consisted of 146 amino acids, the N-terminal portion of the molecule commencing with the sequence CYS-TYR-CYS-. Derynck and others subsequently reported (7) a further recombinant gamma interferon having the same N-terminus and a single amino acid substitution, the polypeptide perhaps constituting an allelic variant of that earlier reported in reference (6). Other workers have reported the production of still further recombinant gamma interferons in which one or more of the amino acids present in Goeddel and Gray's original publication (6) have allegedly been substituted.

[0007] For example, Alton et al (17) report on a series of IFN-gammas wherein a single amino acid substitution at position 81 of the Gray et al (6) gamma interferon resulted in an IFN-gamma that retained only 70 percent of the activity (on a relative basis) and wherein an additional deletion of the cys-tyr-cys at positions 1, 2, 3 of this IFN-gamma further reduced relative activity resulting in an IFN-gamma having only 49 percent of the Gray et al (6) gamma interferon.

[0008] In our hands, recombinant gamma interferons whose N-terminal amino acid sequence comprises cysteine residues have proven problematic from the standpoint of oligomerization which may involve participation of sulphydryl groups of one or more of the cysteine residues in disulfide bond formation. Our inability to completely reduce these putative disulfide linkages suggests the problem may be more complex, possibly also involving reaction through the hydroxyl function of the cysteine-bounded tyrosine residue. These recombinant interferons have proven somewhat unstable and, whether resulting from such instability or otherwise, have proven of less than optimal utility.

[0009] The present invention provides gamma interferon polypeptides modified at the N-terminal and/or C-terminal regions with respect to the sequence disclosed in Gray et al, supra, by in one aspect providing a gamma interferon polypeptide consisting of amino acid sequence, extending from the N-terminus :

X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-LYS-TYR-PHE-  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

5 ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU- cil  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

10 GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

15 LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75

ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-LYS-ARG-ASP-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90

20 ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105

25 GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120

SER-PRO-ALA-ALA-LYS-THR-Z  
 121 122 123 124 125 126

30 wherein:

X is a methionine residue, Y is a glutamine residue and Z consists of n amino acids in the sequence 127-143 depicted in Fig. 1 hereof where n is zero or an integer of from 1 to 17, but excluding as an isolated polypeptide the Lys<sup>78</sup> variant polypeptide of the above-recited sequence where X = methionine, Y = glutamine and Z =

35 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
 127 128 129 130 131 132 133 134 135

40 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN,  
 136 137 138 139 140 141 142 143

45 or the Gln<sup>137</sup> variant thereof; or X is hydrogen, Y is either a glutamine or a pyroglutamate residue and Z consists of n amino acids in the sequence 127-143 depicted in Fig. 1 hereof where n is zero or an integer of from 1 to 16; or a modification or allelic variation of the said amino acid sequence which (modification or variation) exhibits biological activity equivalent to or greater than that of gamma interferon of the amino acid sequence 1-143 depicted in Fig. 1 hereof, but excluding those of the said modifications and variations which contain any gamma interferon amino acid sequence contiguously upstream of Y or contiguously downstream of Z.

[0010] In another aspect the invention provides pharmaceutical compositions comprising such gamma interferon polypeptides. Z may be part of the sequence

55 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
 127 128 129 130 131 132 133 134 135

PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
 136 137 138 139 140 141 142 143

starting with Gly 127, or it may be part or all of said sequence in the case where X is a methionine residue.

[0011] The invention also provides corresponding expression vectors capable of producing such interferon polypeptides, and transformants useful in the production through recombinant DNA technology of the interferon polypeptides of the invention. Preferred embodiments of the invention exhibit greatly improved stability and activity relative to those previously described in the literature.

5 [0012] In the drawings:

[0013] The manner in which these and other objects of the invention are attained will be apparent from the detailed description which follows and from the accompanying drawings, in which:

10 Fig. 1 illustrates amino acids 1 through 143 of a recombinant gamma interferon of the present invention and DNA sequence encoding the amino acid sequence preceded by a signal sequence, which DNA sequence is flanked by regions of 5'- and 3'-untranslated DNAs.

Fig. 2 illustrates schematically a plasmid coding for direct synthesis of a recombinant gamma interferon of the present invention in E. coli and its preparation.

15 Fig. 3 records data demonstrating the enhanced stability of gamma interferon prepared according to the present invention.

20 [0014] We have learned that native human gamma interferon (i.e., that arising from mitogen induction of human peripheral blood lymphocytes and subsequent purification) is a polypeptide which lacks the CYS-TYR-CYS- N-terminus assigned by Gray et al. to the recombinant gamma interferon whose sequence is depicted in (6). Tryptic digests of highly purified native gamma interferon in our hands included sequences whose amino acid composition generally corresponded to that of the N-terminal portion of the Gray et al. recombinant gamma interferon of (6), less CYS-TYR-CYS. Amino acid sequence analysis from the N-terminus of native gamma interferon proved unavailing, giving rise to the inference that the alpha amino acid at the N-terminus of the molecule was protected. Since the first amino acid beyond the second cysteine of Gray et al. (6) for which the cDNA coded was GLN (glutamine), we surmised that cyclization of the GLN residue had left instead pyroglutamate, such that the N-terminus was blocked. Removal of pyroglutamate with pyroglutamate aminopeptidase left a free alpha amino group associated with ASP, the next encoded amino acid, and sequence analysis could proceed, permitting the first reported characterization of native human gamma interferon.

25 [0015] Appropriate alteration of cDNA for CYS-TYR-CYS- containing recombinant human gamma interferon permitted the direct expression in E. coli of novel recombinant gamma interferon from a cDNA encoding the protein whose full sequence is set out supra, X being MET and Y being GLN. The N-terminal methionine is an artifact encoded by the mRNA translational "start" signal AUG which, in the particular case of E. coli expression exemplified, is not processed away by host systems. In other microbial systems, e.g., pseudomonas, methionine may be removed; it does not in any event appear required for activity. Where methionine is removed and depending upon the system employed, the GLN residue may cyclize to the pyroglutamate form, again without any believed impairment of activity.

30 [0016] In our hands, the CYS-TYR-CYS- containing recombinant gamma interferon earlier reported by Gray and co-workers benefited from formulation with human serum albumin in aid of stabilization. The presence of serum albumin in the final lyophilized product, however, requires that certain quality control steps be performed in advance of lyophilization rather than upon finished product. In the case of the novel recombinant gamma interferon of present invention, on the other hand, the material in lyophilized form has proven to be sufficiently stable without the inclusion of serum albumin. Where desired, however, the gamma interferons of the invention may be formulated with pharmaceutically acceptable levels of human serum albumin.

35 [0017] Beyond the foregoing, the CYS-TYR-CYS- lacking recombinant human gamma interferons of the invention appear in cytopathic effect inhibition testing to be markedly more active as antiviral agents than their CYS-TYR-CYS-containing analogs. The activity is conventionally assayed in microtiter plates by inhibition of the cytopathic effect (CPE) of encephalomyocarditis virus on human lung carcinoma cells A549. See (12).

40 [0018] The recombinant gamma interferons of the invention include all those comprising amino acids 1 to about 126 of the full sequence provided above. Gamma interferons variously truncated at the carboxy terminal end relative to the full sequence continue to exhibit the characteristic properties of human gamma interferon, albeit at diminished levels in some cases, so long as amino acids 1 to about 126 are present. Indeed, experiments with the CYS-TYR-CYS-containing analog reported at (7) showed that extraneous sequences could be substituted for the amino acid sequence following amino acid 132 (by the present numbering system) without loss of activity. See, e.g., (8). Preliminary evidence in our hands supports the hypothesis that while amino acids 1 to about 126 (THR) are relatively tightly bound in a three-dimensional configuration we associate with activity, remaining amino acids of the full sequence are by comparison less confined and relatively sensitive to proteolysis. Trypsin digestion under limiting conditions removes various portions of the sequence downstream from amino acid 126, but not upstream therefrom. Native gamma interferon moieties in our hands include molecules variously extending through amino acids 127, 128, 129, 130, 132 and 134. We have seen

fully active recombinant gamma interferon whose amino acid sequence following methionine consisted variously of amino acids (beyond MET) 1 to about 139 and 1 to about 131, the latter obtained by limited digestion of recombinant gamma interferon with trypsin followed by sequence confirmation. Similar trypsin digested fragments variously ending at about amino acids (beyond MET) 128 and 129 retained activity, albeit substantially diminished. On the other hand, material having 125 amino acids (in addition to N-terminal methionine), the threonine at position 126 and following amino acids having been digested away, exhibited less than 1% the activity of undigested material in CPE inhibition assay.

[0019] Recombinant derived gamma interferon, in addition to its bearing an initial methionine when produced in hosts where the methionine is not intracellularly cleaved, appears to exhibit a major species having 139 amino acids (based on the numbering system of Figure 1) and a minor species having 143 amino acids. The composition of the two species contains greater than about 95 percent, most preferably greater than about 97 percent of the species having 139 amino acids. Trypsin digestion under limiting conditions likewise removes various portions of the sequence downstream from about amino acid 126. Recombinant gamma interferon in our hands following such limiting trypsin digestion include species variously extending through amino acids 125, (126 with an initial methionine) 129, 131 and 134. These species have retained activity, albeit substantially diminished, in the zone extending from about amino acid 125 to about 129. Species having at least about 129 amino acids, and especially at least about 131 amino acids, i.e., species having from about 129 to 143 amino acids are essentially functionally fully active.

[0020] By reference to "recombinant gamma interferon" herein we intend a polypeptide (whether or not glycosylated by the cell in which it is produced) expressed in a transformant cell from a replicable expression vehicle arising from recombinant DNA technology, the polypeptide exhibiting in greater or less degree the antiviral and antiproliferative activity in humans and pH 2 labile properties characteristic of native human gamma interferon.

[0021] The recombinant gamma interferons as claimed are believed to form "dimers", defined for the purpose of this disclosure as a combination of two such polypeptides (which polypeptides may have the same or a different number of amino acids). The nature of the chemical combining mechanism is not fully understood, but is believed to be other than covalent bonding. This combination into dimers appears to occur spontaneously and is believed to be inevitable in the systems described herein. Thus, when the recombinant gamma interferons of the present invention are administered, they will usually be in the dimerized form.

[0022] Furthermore, subject to the terms of the appended claims, it is to be understood that here, similarly to the disclosures in the literature relating to differently characterized recombinant gamma interferons (8, 9), amino acid substitutions or additions, particularly single amino acid substitutions and addition or substitution of groups of amino acids upstream or downstream from about amino acid 126 in the recombinant gamma interferons disclosed herein are possible without destroying the interferon activity of the polypeptides from which such modifications are derived. It is believed that it will be obvious to those skilled in the art to make such substitutions or additions without departing from the scope of the present invention claimed. Again, the recombinant gamma interferons of the present invention as claimed include species having modifications and allelic variations of the full sequence (see Figure 1) which exhibit biological activity equivalent to or greater than that of the full sequence.

[0023] Characteristically, purified recombinant gamma interferons will be essentially free of other proteins of human origin, and are so to be distinguished from the native human gamma interferon compositions heretofore available.

[0024] The invention includes recombinant gamma interferon compositions which (prior to formulation) are greater than about 95 percent pure, preferably more than about 98 percent pure, which compositions are for that reason likewise distinct from native gamma interferons heretofore available.

[0025] Embodiments of the invention as claimed in which the N-terminal amino acid residue is methionyl are likewise distinct from gamma interferons produced within the human body, and appear also for that reason to be distinct, beyond their deletion of CYS-TYR-CYS-, from those whose sequence is reported by Gray et al. (6).

[0026] The replicable expression vehicles referred to herein are double-stranded DNA moieties, preferably plasmids, comprising an origin of replication, a promoter or promoter-operator, a sequence encoding a ribosome binding site, a codon for a translation start signal, and in proper reading phase therewith a gene encoding the recombinant gamma interferon of interest, followed by codon(s) for a translation stop. At the present stage the general techniques and lexicography of recombinant DNA technology are well understood to the art-skilled, who are referred in any event to (11) for background information pertinent to the practice of the present invention, mutatis mutandis, in all its embodiments and legally cognizable equivalents.

#### Example

##### 55 A. Cloning

[0027] Recombinant DNA clones containing gamma interferon cDNA sequences were prepared as described in (6) and in the aforementioned GB 2107718 with messenger RNA from induced human peripheral blood lymphocytes. The

DNA sequence of clone p67 is shown in Figure 1. A 5' untranslated region is followed by 69 nucleotides encoding a precursor or signal peptide of 23 amino acids, 429 nucleotides coding for a mature interferon polypeptide of 143 amino acids, and 587 nucleotides of 3' untranslated sequence.

5      B. Expression

1      1. E.Coli Example

[0028] To express high levels of recombinant IFN- $\gamma$  in E. coli, the initiation of protein synthesis must occur at an ATG codon immediately preceding the glutamine codon (amino acid one) of the mature polypeptide rather than at the ATG of the signal peptide (amino acid S1) (Fig. 1). The procedure followed to express the cDNA insert of p67 directly in E. coli is outlined in Fig. 2. The approach was similar to that used to express in E. coli the cDNA insert of Gray et al. (6).

[0029] An Aval restriction site located at codon 2 of the presumed mature coding sequence was utilized to remove the signal peptide coding region. Two synthetic deoxyoligonucleotides were designed which restore the codons for amino acids 1 and 2, incorporate an ATG translational initiation codon, and create an XbaI cohesive terminus. These two oligomers were ligated to the remainder of the cDNA insert to construct a 1063 base-pair synthetic-natural hybrid gene coding for a polypeptide of 144 amino acids and bounded by XbaI and PstI sites. This gene was inserted into the plasmid pLeIF A25 (10) between the XbaI and PstI sites to give the expression plasmid py-CYC5. E. col strain W3110 (F-,  $\lambda$  -, protrophic) (ATCC No. 27325) was transformed with this plasmid to give the host-vector combination E. coli W#110/py-CYC5.

2      2. Cell Culture Example

[0030] Expression of a gene encoding both the signal peptide and gamma interferon, as indicated in Figure 1, was effected in COS-7 cells (16) in the presence of radioactively labeled cysteine and methionine, confirming the production from the gene of mature gamma interferon whose N-terminal amino acids are as indicated in Figure 1 (unlike the case involving E. coli expression, the expression product of mammalian cell systems like that exemplified here lacks N-terminal methionine).

[0031] Confluent monolayers of COS-7 cells in 60 mm petri dishes were transfected in duplicate with DNA using the modified DEAE-dextran procedure. Three days after DNA addition, the media was removed. Each set of plates received 2 mls DMEM supplemented with either 100  $\mu$ Ci S<sup>35</sup>-methionine or S<sup>35</sup>-cysteine. After 16 hours incubation in the presence of the radiolabeled amino acid; the media was removed and 500  $\mu$ l immunoprecipitated using an anti-gamma-interferon monoclonal antibody or an anti-HBsAg monoclonal antibody as the first antibody and a rabbit anti-mouse IgG antibody (Cappell Inc.) as the second antibody. Reaction with the antibody and the subsequent binding to Staphylococcus A cells (Calbiochem) are as described by Berman, P. et al. (18). The samples were resuspended in SDS-mercaptoethanol and electrophoresed on 10 $\Delta$  SDS-PAGE gels. The gel was fixed in 7 $\Delta$  acetic acid in ethanol, soaked in Enhance (New England Nuclear) fluor solution, dried down, and exposed for two weeks using Kodak AR5 film and an intensifying screen (Dupont).

[0032] Plasmids used in this study were pSVgamma69 (11); pDL RI (19), a hepatitis B virus surface antigen expression vector upon which pSVgamma69 was based; and pDL Rlgamma Sau, a polycistronic plasmid containing the 830 bp SAU3a fragment of pSVgamma69 (11) spanning the entire gamma-interferon encoding sequences inserted into the EcoRI site of pDL RI. The latter plasmid produces a transcript containing both the gamma-interferon and the HBsAg coding regions.

[0033] Comparison of S<sup>35</sup>-Cysteine and S<sup>35</sup>-Methionine labeled proteins which react with either anti-gamma-interferon (A) or anti-HBsAg (B) antibodies showed that no material migrating at either the glycosylated (29,000 MW) or monoglycosylated position (18,000 MW) was specifically immunoprecipitated from S<sup>35</sup>-Cys labeled pDL RI-gamma Sau transfected cells using anti-gamma-interferon antibody, in contrast to the S<sup>35</sup>-met labeled cells which showed the immuno-precipitation of interferon-gamma.

50     C. Fermentation Production

[0034] The production of recombinant Human Interferon - Gamma (rIFN- $\gamma$ ) using E. coli W3110/py- CYC5 is carried out in batches ranging in volume from 10 to 1000 liters. After fermentation, the interferon containing E. coli cells are recovered from the broth for isolation and purification of rIFN- $\gamma$ . The following is a description of the fermentation and cell recovery processes.

1. Preparation and Maintenance of Stock Cultures

[0035] A stock culture is prepared in sterile baffled culture flasks containing 150 to 500 mL of a sterile medium having the following composition.

5      Bactotryptone 10 g/L  
 Yeast Extract 5 g/L  
 Sodium Chloride 5-10 mg/L

10     The medium is then inoculated with a primary culture of E. coli 143110/py -CYC5. The inoculated flask is then incubated on a shaker at 25-37° C until the absorbance at 550 nm reaches approximately 1.0. Approximately 50 percent v/v of 30 percent v/v dimethyl sulfoxide is added to the broth. One (1) mL aliquots are immediately dispensed into sterile vials and capped. The vials are stored at -60°C or below. Each fermentation is started using a replicate stock culture for inoculum.

15     2. Inoculum Preparation

[0036] The inoculum is prepared in the medium previously described (L.B. Broth) in either shaker flasks or small fermenters. After incubation at about 37°C for approximately 8 hours, the inoculum is transferred to a fermenter. The 20 volume of the inoculum is between 2 to 10 percent of the volume of the fermentation.

3. Fermentation

[0037] Recombinant Interferon - Gamma production is carried out in fermenters with working volume of about 10 to 25 1000 liters. The fermentation medium is composed of:

		Per liter
30	*Glucose	50-100 g
	Ammonium Sulfate	4.0-8.0 g
	Potassium Phosphate, Monobasic	3.0-5.0 g
	Potassium Phosphate, Dibasic	5.0-8.0 g
	Magnesium Sulfate, Heptahydrate	0.5-5.1 g
	Sodium Citrate, Dihydrate	0.5-2.0 g
35	UCON LB-625	0.5-2.0 mL
	Ferric Chloride, Hexahydrate	0.005-0.15 g
	Zinc Sulfate, Heptahydrate	0.001-0.15 g
	Cobalt Chloride, Hexahydrate	0.001-0.005 g
40	Sodium Molybdate, Dihydrate	0.001-0.005 g
	Cupric Sulfate, Pentahydrate	0.001-0.005 g
	Boric Acid	0.001-0.005 g
	Manganese Sulfate, Monohydrate	0.001-0.005 g
	Hydrochloric Acid	0.0-1.0 mL
45	Thiamine-HCl	0.0-0.1 g
	Tetracycline HCl	0.001-0.01 g
	*L-Tryptophan	0.1-0.5 g
	Yeast Extract	2.0-8.0 g
50	3-β-Indoleacrylic acid	0.02-0.10 g

\*A portion of the glucose and tryptophan is added to the fermenter initially and the remainder is fed throughout the fermentation process.

[0038] Ingredients in the medium are sterilized by heat treatment or filtration prior to use in fermentation. The fermentation is carried out at 25-40°C Other operating conditions are as follows:

55

Agitation (rpm)	100-1000	
Aeration (wm)	0.5-1.5	(Supplemented with oxygen when necessary)

(continued)

pH	6.5-7.5	(Controlled by the addition of ammonium hydroxide)
----	---------	--

5    4. Purificationa. Extraction of Recombinant Gamma Interferon.

[0039] *E. coli* cells are suspended in a medium which contains salts and an appropriate buffer in the pH range of 6 to 9, preferably about 9. Recombinant gamma interferon is extracted by homogenization of the cell suspension in a high pressure colloid mill such as a Gaulin mill. Sufficient polyethylenimine is added to the solution to produce a 0.1 to 1% W/V solution. The supernatant contains gamma interferon.

b. Partial Purification of Recombinant Gamma Interferon on a Silica-based Adsorbant.

[0040] The supernatant from part (a) is adsorbed to a silica based adsorbant which is washed to remove impurities with appropriate salt solutions in the pH range of 6 to 9. Recombinant gamma interferon is eluted using a solution containing 0.5 - 1.0 M tetramethyl ammonium chloride. All operations in this step are performed in the pH range of 7 to 9.

20    c. Partial Purification of Recombinant Gamma Interferon by Anion Exchange Chromatography

[0041] The eluent from part (b) is dialysed and adsorbed to an anion exchange chromatography medium which is then washed to remove impurities. Recombinant gamma interferon is eluted with a gradient of increasing salt. Typical anion exchange resins applicable for this step include carboxymethyl cellulose and sulphoethyl cellulose. All operations are performed in the pH range of between 7 and 9.

25    d. Partial Purification of Recombinant Gamma Interferon by Chromatography on Calcium Phosphate Gel

[0042] The eluent from part (c) is adsorbed to a medium of calcium phosphate which is then washed to remove impurities. The recombinant gamma interferon is eluted by increasing the salt concentration in a gradient of increasing phosphate concentration. All operations in this step are performed in the pH range of between 7 and 9.

30    e. Partial Purification of Recombinant Gamma Interferon by Anion Exchange Chromatography

[0043] The eluent from part (d) is dialysed and adsorbed to an anion exchange chromatography medium which is then washed to remove impurities. The recombinant gamma interferon is eluted from the anion exchange medium with a gradient of increasing salt concentration. Typical anion exchange chromatography media are carboxymethyl cellulose and sulphoethyl cellulose. All operations in this step are performed in the pH range of between 7 and 9.

35    f. Partial Purification of Recombinant Gamma Interferon by Gel Permeation Chromatography.

[0044] The eluent from part (e) is applied to a gel permeation medium and the column is developed with a salt containing medium. The appropriate recombinant gamma interferon containing fractions are pooled to produce the bulk drug substance. All operations in this step are performed in the pH range of between 7 and 9.

40    9. C-Terminal Amino Acid Sequence

[0045] To determine the c-terminal sequence samples were dialyzed into 70 percent formic acid, cleaved with cyanogen bromide and the resulting peptides separated on an Altex Ultrasphere C8 reverse phase HPLC column. Peaks were collected and analyzed by amino acid and sequence analysis. One C-terminal peptide was found: - leu-phe-arg-gly-arg (residues 135-139, Figure 1). In some cases another additional peptide was detected: -leu-phe-arg-gly-arg-arg-alanine-ser-gln (residues 135-143, Figure 1). To determine the ratio of these two peptides, known amounts (by amino acid analysis) were loaded onto the reverse phase HPLC column and the respective peak heights determined. Each of three production lots contained less than about two percent of the long peptide (135-143, Figure 1), the balance being the 5-mer. This data is consistent with *E. coli* production of a mixture of 139 amino acid-containing and 143 amino acid-containing gamma interferons (excluding the N-terminal methionine, which is also present in each case) in the relative proportions, respectively, of about 98.2 percent.

5. Formulation

[0046] Recombinant gamma interferon made in accordance with the foregoing is preferably formulated for parenteral administration according to the following Table.

5

10

15

20

25

	Ingredient	QUANTITY PER VIAL	
		0.5 mg vial(1)	2.0 mg vial(2)
	Recombinant Human Interferon - Gamma	0.5	2.0
	Mannitol	100	80
	Succinic Acid Disodium Hexahydrate	12.4	9.9
	Glycine	5.6	4.5
	Sodium Chloride	4.4	3.5
	Polysorbate 20	0.8	0.6
	Succinic Acid	0.5	0.4

(1) Vials are reconstituted with 2.5 ml sterile Water for Injection.

(2) Vials are reconstituted with 2.0 ml sterile Water for Injection.

30

[0047] The interferons of the invention may be employed in medically appropriate dosage ranges, e.g., 1.0 mg/M<sup>2</sup> of body surface area.

D. Determination of the Activity of Various Gamma Interferons Following Trypsin Digestion

35

[0048] To establish the activity of various gamma interferons differing in their carboxy-termini gamma interferon prepared as described in the *E. coli* example, supra, was digested with trypsin to various degrees and tested by CPE assay with A549 cells as described within.

40

[0049] A sample of recombinant gamma interferon (r-HuIFN-gamma) (6.5 mg), prepared as described herein, was desalted over a small Sephadex G-25 molecular sieving column (PD-10, Pharmacia) into 0.10 M Ammonium Bicarbonate buffer, pH 8.5 to a final protein concentration of 2.1 mg/ml. A dilute trypsin solution (Worthington TPCK trypsin, 10 µg/ml in 0.001 M HCl, 16 µl) was added to 1.9 ml (4.0 mg) of the r-HuIFN-gamma solution, mixed and incubated at room temperature (trypsin:protein:1:25,000). Samples were removed from the incubation mixture at 1 hr., 3.5 hrs., 5.75 hrs., 8 hrs. and 10.25 hrs. At 8 hrs., an additional 15 µl (150 ng) of dilute trypsin solution was added in order to accelerate the reaction for the last time point sample at 10.25 hrs.

45

[0050] Fractionation of each time point sample into its respective components was performed on a Waters HPLC system using a BioRad Biogel HPHT Column. The time point aliquot in bicarbonate buffer was loaded directly (by manual injection) to the column at the time of sampling. The column was equilibrated in 0.01 M sodium phosphate pH 8.0, 30 µM calcium chloride and protein was eluted from the column utilizing a linear gradient of the equilibration buffer and 0.5 M sodium phosphate buffer pH 8.0, 0.6 µM calcium chloride.

50

[0051] Protein peaks as determined by absorbance at 214 nm and 280 nm were preparatively collected and stored covered at 4°C until analyzed. Typical analyses for selected peaks included:

1. Antiviral activity in the human lung carcinoma A549/EMC virus assay system (13).
2. SDS/PAGE fractionation by standard techniques employing the Laemmli gel system (14).
3. Protein concentration determination by the commercial (Pierce Chemical Co., Rockford, IL.) dye binding procedure.

55

## 4. Cyanogen bromide protein cleavage and subsequent HPLC analysis for peptide identification (15).

Samples of protein are dialyzed (12,000-14,000 mw cut off) overnight against 70% formic acid, taken to dryness by rotary evaporation and resuspended in 500 µl of 70% formic acid. Solid cyanogen bromide is added to each sample in a 12×75 mm glass tube, sealed, mixed until dissolved, covered with aluminum foil and incubated overnight at room temperature in a well ventilated area.

[0052] After cleavage, samples are taken to dryness by rotary evaporation, resuspended in 0.5 ml of water and redried. Prior to fractionation by HPLC, samples were redissolved in 50% formic acid to a protein concentration of approximately 1 mg/ml.

[0053] Peptides were fractionated using a Waters HPLC system employing an Altex Ultrasphere Octyl column and a trifluoroacetic acid/water - trifluoroacetic acid/acetonitrile linear elution gradient. Wherever possible, peptides were identified by amino acid analysis. Table I lists comparative data for shortened forms of r-HuIFN-γ:

Table 1

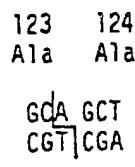
	r-HuIFN-γ Form*	C-Terminus**	Specific Activity (%)
15	139aa:143aa::98:2 (for comparison)	LFRGR	100
	131aa	AAKTGKRKR	40-50
20	129aa	AAKTGKR	6-9
	125aa	AAK	ca. 1

\* Based on numbering system of Figure 1, but excluding the N-terminal methionine also present in each case.

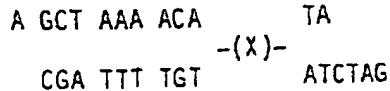
\*\* Conventional single letter abbreviations for amino acid residues:

- A = alanine
- F = phenylalanine
- G = glycine
- K = lysine
- L = leucine
- R = arginine
- T = threonine

[0054] It will be appreciated that gamma interferons of any length within the range 126aa-143aa (excluding N-terminal methionine) will be expressed from appropriately tailored genes. Thus, for example, the gene depicted in Figure 1 contains a Fnu4H restriction site at



45 Following restriction with Fnu4H, synthetic oligonucleotides encoding any desired sequence followed by a "stop" codon and a linker compatible with an available restriction site in the expression plasmid may be ligated to the fore part of the gamma interferon gene. For example, the sequence



50 wherein X encodes one or more amino acids may be ligated into the *E. coli* expression vehicle exemplified above following digestion of the plasmid with Fnu4H and BgIII, a stop codon resulting from ligation thusly

stop  
... (X)-TAGATC...  
ATCTAGI  
BglII

5

E. Assay: Inhibition of Cytopathic Effect (CPE)

## 10    1. Test Procedure

[0055] To each well add 100 microliters of a suspension of human lung carcinoma (A549) cells (ATCC No. CCL 185) which have been adjusted to contain  $4 \times 10^5$  cells/ml in Eagles MEM.

[0056] Incubate plates at 37°C for approximately 18 hours.

15 [0057] After 18 to 24 hours incubation, add to each well in the first column, 80 microliters additional medium.

[0058] Add to a well in the first column 20 microliters of a sample to be assayed for interferon activity.

[0059] Transfer 100 microliters of the contents of each well in the first column horizontally to each well in second column.

20 [0060] Continue to transfer 100 microliters of the contents of a well from column to subsequent column until a total of 10 transfers are performed ending in column 11.

[0061] After 24 hours of incubation, challenge all wells, except cell controls, with 50 microliters of encephalomyocarditis virus at a multiplicity of infection which results in 100 % cytopathic effect in 24 hours after infection.

Cover trays with lids and incubate at 37°C for 24 hours.

Pour off fluid from all wells and stain 5-15 minutes with 0.5% crystal violet.

25 Viability of cells is determined by observation of stained cells.

Titer of a sample is the reciprocal of the dilution where 50% viable cells remain.

2. Calculation

30 [0062] The activity of all samples is normalized by the Reference Units Conversion Factor which is calculated from:

$$\frac{\text{Actual Titer of Standard Interferon}}{\text{Observed Titer}} = \text{Reference Units Conversion Factor} = \text{RUCF}$$

## 35    3. Specific Activity

[0063] Using the A549/EMCV bioassay systems standardized with IFN-gamma reference material provided by the NIH, the specific antiviral activity of recombinant human IFN-gamma is approximately three times higher than the activity of the modified rIFN-gamma molecule with three additional amino acids (cys-tyr-cys) at the N-terminus.

40

## 4. Stability

[0064] Based again on the above mentioned measure of bioactivity (A549/EMCV), formulated and vialled rIFN-gamma continues to be stable (no loss of biological activity) three months after production (stored at 4°C).

45

F. Antiproliferative activity of rIFN-gamma compared to the other types of human interferon

## 1. Materials and Methods

## 50    [0065]

rIFN-gamma: Des Cys-Tyr-Cys recombinant interferon gamma in the form of a solution (20 mM sodium succinate, 0.15 M NaCl, pH 6). The material was prepared in accordance with the E. coli Example above. The specific activity was  $2.7 \times 10^7$  IU/mg protein.

55 CTC-rIFN-gamma: Recombinant interferon gamma having "Cys-Tyr-Cys" structure at the N-terminus of the molecule in the form of a solution (20 mM sodium succinate, 0.15 M NaCl, pH 6). The specific activity was  $1.3 \times 10^7$  IU/mg protein.

HuIFN-beta: Lyophilized human fibroblast interferon produced in human diploid foreskin fibroblast with a spe-

cific activity of more than  $1 \times 10^7$  IU/mg protein prepared by Toray Ind., Inc. A vial contains  $3 \times 10^6$  IU HulFN-beta and 3 mg human serum albumin.

HulFN-alpha: Human natural leukocyte interferon with a specific activity of  $4 \times 10^6$  IU/mg protein supplied in the form of solution by Dr. K. Cantell, Central Public Health Laboratory, Helsinki, Finland.

5 Control: Placebo containing 3 mg human serum albumin.

Culture Medium: Eagle's minimum essential medium supplemented with 10 percent heat-inactivated precolostrum newborn calf serum ("PNCS") and 2 mM L-glutamine was used with HeLa, KB, HMV-I, FL, and J-III cells. Dulbecco's minimum essential medium containing 10 percent heat-inactivated PNCS, 100 µg/ml kanamycin, and 2 mM L-glutamine was used with A549 cells. RPMI 1640 medium 10 supplemented with heat-inactivated PNCS and 100 µg/ml kanamycin was used with the remaining human cells listed in Table 2.

## 2. Evaluation of antiproliferative activity

15 [0066] The test cells suspended in the culture medium were seeded into plastic tissue culture plates at a concentration of  $5 \times 10^3$  cells/0.5 ml/well. Various amounts of interferon dissolved in the corresponding culture medium (0.5 ml) were added subsequently (day 0). Cultivation was carried out at 37°C in a humidified atmosphere of 5 percent CO<sub>2</sub> and 95 percent air. On day 6 the culture media were removed and the cells in suspension culture were directly suspended in Isoton II (Coulter Electronics Inc.) for cell counting in a Coulter counter. The cells forming a sheet in plastic vessels 20 were pre-treated with 0.05 percent trypsin-0.02 percent EDTA to prepared single cell suspension in Isoton II (Coulter Electronics Inc.). Antiproliferative activity of interferon was expressed as the antiviral units required to produce 50 percent reduction of cell number (IC<sub>50</sub>, IU/ml) compared to the control culture (without interferon).

25 [0067] As shown in the table, the antiproliferation activity of rIFN-gamma varied markedly depending on the human cell species. In this case KATO-III, siglet-ring cell carcinoma of the stomach, was highly sensitive, the cell line showing the IC<sub>50</sub> of 1.2 IU/ml, while Daudi cells, Burkitt's lymphoma, which were highly sensitive to type II interferon (HulFN-alpha, HulFN-beta), were insensitive to type II interferon including rIFN-gamma. Lung adenocarcinoma (PC-8, PC-12) was insensitive to all interferon species tested. The anticellular spectrum between rIFN-gamma and CTC-rIFN-gamma was almost the same and it was generally apparent that the antiproliferative efficacy of rIFN-gamma was superior to that of CTC-rIFN-gamma. In the case of comparison between four interferons, the highest efficacy was obtained from 30 rIFN-gamma except for Daudi cells.

## G. Comparison of stability between rIFN-gamma and CTC-rIFN-gamma in various fluids in vitro

35 [0068]  $1 \times 10^6$  IU/vial of lyophilized rIFN-gamma prepared in accordance with the E. coli Example above and  $1 \times 10^6$  IU/vial of lyophilized rIFN-gamma having a "Cys-Tyr-Cys" structure at the

40

45

50

55

TABLE 2  
ANTIPIROLIFERATIVE ACTIVITY OF rIFN- $\gamma$  COMPARED TO OTHER INTERFERON SPECIES

Cell	Histological Type or Origin of Test Cells	IC50 (IU/ml)			
		rIFN- $\gamma$	CTC-rIFN- $\gamma$	HuIFN- $\beta$	HuIFN- $\alpha$
KATO-III	Siglet-ring cell carcinoma of stomach	1.2	1.3	--	--
MKN-1	Adenosquamous carcinoma of stomach	37	330	--	--
MKN-28	Well differentiated adenocarcinoma of stomach	$>10^3$	$>10^3$	--	--
MKN-45	Poorly differentiated adenocarcinoma of stomach	$>10^3$	$>10^3$	--	--
MKN-74	Well differentiated adenocarcinoma of stomach	$>10^3$	$>10^3$	--	--
HeLa	Uterine cervix carcinoma	22	132	446	$>10^3$
KB	Nasopharyngeal carcinoma	54	330	$>10^4$	$>10^4$
HMV-1	Amelanotic melanoma	38	45	130	820
SEK1-F	Melanoma	135	$>10^3$	137	663
FL	Aminion (non-malignant origin)	38	45	$>10^3$	$>10^4$
PC-8	Lung poorly differentiated adenocarcinoma	$>10^3$	$>10^3$	$>10^4$	$>10^3$
PC-12	Lung adenocarcinoma	$>10^3$	$>10^3$	$>10^4$	$>10^3$
A549	Lung alveolar carcinoma	55	--	--	--
QG56	Lung squamous cell carcinoma	5	--	--	--
QG90	Lung anaplastic small cell carcinoma	5.5	--	--	--
Daudi	Burkitt's lymphoma	$>10^3$	$>10^3$	47	7
Namaiwa	Burkitt's lymphoma	$>10^3$	$>10^3$	--	--
J-111	Monocytic leukemia	25	84	204	$>10^3$

N-terminus, with 10 mg human serum albumin, phosphate buffer and an isotonic amount of NaCl for each interferon were reconstituted with distilled water and the concentration was adjusted to  $4 \times 10^4$  IU/ml.

[0069] In vitro stability of interferons was evaluated by the determination of the residual anti-viral activity in various fluids. Incubations were initiated by addition of the above interferon solutions into nine volumes of rabbit serum, human serum or Eagle's MEM which were pre-incubated 10 min, in a water bath incubator at 37°C or 4°C. At 0, 0.25, 0.5, 1, 4, 8, 24, 72 and 144 hr., an aliquot was harvested and mixed with the nine volumes of Eagle's MEM. The samples 5 were kept frozen at -80°C in a deep freezer until the assay of interferon titer. Interferon titer was assayed by the CPE50 reduction method using human amniotic cells (FL cell) challenged with Sindbis virus. The results are indicated in Figure 3 as percentages of residual titer against the additional titer.

[0070] While the invention has been exemplified by reference to the preferred embodiments in which expression is effected in E. coli and in COS-7 cells, it will be apparent that the gamma interferons as claimed may be produced in 10 other systems as well such as other bacterial strains, yeast and tissue culture systems, as to which see GB 2107718 and (6). Thus, the invention is not confined to the most preferred embodiments, but instead extends to all legally cognizable equivalents of the claims which follow.

#### Bibliography

15 [0071]

1. Goeddel, D. et al., Nature 287, 411 (1980)
2. Goeddel, D. et al., Nature 290, 20 (1981)
- 20 3. Yelverton, E. et al., Nucleic Acids Research 9, 731 (1981)
4. Guterman et al., Annals of Int. Med. 93, 399 (1980)
5. Goeddel, D. et al., Nucleic Acids Research 8, 4057 (1980)
6. Gray, P. et al., Nature 295, 503-508 (1982)
7. Derynck, R. et al., Nucleic Acids Research 10, 3605 (1982)
- 25 8. Derynck, R. et al., Interferon Scientific Memoranda, August 1982, Memo-I-A1193/2.
9. Derynck, R. et al., "Expression of Human Interferon Gamma in Heterologous Systems" in Experimental Manipulation of Gene Expression, Academic Press, Inc. (1983) at 247
10. Goeddel, D. et al., Nature 287, 411-416 (1980)
11. European Patent-Application of Goeddel, D. et al., EPO publication No. 0 077 670.
- 30 12. W. E. Stewart II in "The Interferon System" Springer Verlag (New York) pp. 13-26 (1979).
13. Stewart, "Interferon Systems" Ed.: Stewart, p. 13 Springer-Derlag, New York (1979)
14. Laemmli, Nature 227, 680 (1970)
15. Gross, et al., Methods in Enzymology, XI, 238
- 35 16. Gluzman Cell 23, 175 (1981)
17. Alton et al., "Production, Characterization and Biological Effects of Recombinant DNA Derived Human IFN-alpha and IFN-gamma Analogs," The Biology of the Interferon System, DeMaeyer and Schellekens, Eds., Elsevier Science Publ. (1983).
18. Berman et al., Science 222, 524 (1983)
19. Simonsen et al., Mol. Cell. Biol. 3, 2250 (1983)

40

#### Claims

45 Claims for the following Contracting States : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. A gamma interferon polypeptide consisting of the amino acid sequence, extending from the N-terminus

50

55

X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-LYS-TYR-PHE-  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15  
 5 ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU-  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
 GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
 10 GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60  
 LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
 15 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-lys-ARG-ASP-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 20 ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 25 SER-PRO-ALA-ALA-LYS-THR-Z  
 121 122 123 124 125 126

wherein:

x is a methionine residue, Y is a glutamine residue and Z consists of n amino acids in the sequence 127-143 depicted in Fig. 1 hereof where n is zero or an integer of from 1 to 17, but excluding as an isolated polypeptide the Lys<sup>78</sup> variant polypeptide having the above-recited sequence where X = methionine, Y = glutamine and Z =

35 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
 127 128 129 130 131 132 133 134 135  
 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN ,  
 40 136 137 139 139 140 141 142 143

or the Gln<sup>137</sup> variant thereof; or x is hydrogen, Y is either a glutamine or a pyroglutamate residue and Z consists of n amino acids in the sequence 127-143 depicted in Fig. 1 hereof where n is zero or an integer of from 1 to 16; or a modification or allelic variation of the said amino acid sequence which (modification or variation) exhibits biological activity equivalent to or greater than that of gamma interferon of the amino acid sequence 1-143 depicted in Fig. 1 hereof, but excluding those of the said modifications and variations which contain any gamma interferon amino acid sequence contiguously upstream of Y or contiguously downstream of Z.

2. A polypeptide according to claim 1 wherein X is methionine.  
 50  
 3. A polypeptide according to claim 2 wherein Z is part or all of the sequence:

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
127 128 129 130 131 132 133 134 135

5 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
136 137 138 139 140 141 142 143

starting with GLY 127.

10 4. A polypeptide of claim 1, wherein Z comprises the sequence:

15 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG  
127 128 129 130 131 132 133 134 135 136 137 138 139

20 5. A polypeptide of Claim 1, wherein Z comprises the sequence:

25 GLY-LYS-ARG  
127 128 129

30 6. A polypeptide of Claim 1, wherein Z comprises the sequence:

35 7. A replicable expression vehicle capable, in a transformant microorganism or cell culture, of expressing a recombinant gamma interferon polypeptide as defined in any one of claims 1 to 6.

8. An expression vehicle according to claim 7 for use in a bacterium.

35 9. A microorganism or cell which has been transformed with the replicable expression vehicle of claim 7 or claim 8

40 10. A process which comprises culturing a transformed microorganism or cell culture of claim 9 to produce said gamma interferon polypeptide.

45 11. A composition suitable for pharmaceutical administration comprising a gamma interferon polypeptide of any one of claims 1 to 6.

12. A composition of claim 11 comprising a mixture of polypeptides of claim 1, in one of which Z is the sequence Gly 127 to Glu 143 and in another of which Z is the sequence Gly 127 to Arg 139, said two sequences being as set forth above.

50 13. A composition of claim 12 wherein the polypeptide in which Z is Gly 127 to Arg 139 is in a major amount relative to the polypeptide in which Z is Gly 127 to Glu 143.

14. A composition of claim 12 wherein the polypeptide in which Z is Gly 127 to Arg 139 comprises greater than about 95% of the polypeptide mixture.

55 15. A composition of claim 12 wherein the polypeptide in which Z is Gly 127 to Arg 139 comprises greater than about 97% of the polypeptide mixture.

16. A composition of any one of claims 12 to 15 wherein X is methionine.

17. A composition according to any one of claims 11 to 16 comprising also a pharmaceutically acceptable level of human serum albumin.
- 5 18. A mixture of gamma interferon polypeptides comprising: (i) a polypeptide of claim 1 in which n is 13, and (ii) a polypeptide of claim 1 in which n is 17.
19. A gamma interferon mixture according to claim 18 wherein species (i) is in a major amount relative to species (ii)
- 10 20. A gamma interferon mixture according to claim 19 wherein the composition of the two species of gamma interferon contains greater than about 95% of species (i).
21. A gamma interferon mixture according to claim 20 wherein said ratio is greater than about 97% of species (i).
22. A gamma interferon mixture according to any one of claims 18 to 21, wherein X is methionine.
- 15 23. A gamma interferon mixture according to any one of claims 18 to 22 wherein in species (i) Z is the amino acid sequence Gly 127 to Arg 139 as set forth above.
- 20 24. A gamma interferon mixture according to any one of claims 18 to 23 wherein in species (ii) Z is the amino acid sequence Gly 127 to Gln 143 as set forth above.
- 25 25. A gamma interferon mixture according to claim 20 wherein X comprises methionine, Z in species (i) is the amino acid sequence Gly 127 to Arg 139 as set forth above, and z in species (ii) is the amino acid sequence Gly 127 to Gln 143 as set forth above.
- 30 26. A gamma interferon mixture according to claim 18 as obtainable by expression in E.coli of recombinant DNA encoding the amino acid sequence 1 to 143 as set forth above, immediately preceded by an ATG translational start codon and followed by a translational stop codon.

**Claims for the following Contracting State : AT**

1. A process which comprises culturing a microorganism or cell culture which has been transformed with a replicable expression vehicle so as to express a gamma Interferon polypeptide consisting of the amino acid sequence, extending from the N-terminus:

40

45

50

55

X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-TYR-PHE-  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15  
 5 ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU-  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
 GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
 10 GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60  
 LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
 15 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-LYS-ARG-ASP-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 20 ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 25 SER-PRO-ALA-ALA-LYS-THR-Z  
 121 122 123 124 125 126

wherein:

30 X is a methionine residue, Y is a glutamine residue and Z consists of n amino acids in the sequence 127-143 depicted in Fig. 1 hereof where n is zero or an integer of from 1 to 17, but excluding as an isolated polypeptide the Lys<sup>78</sup> variant polypeptide having the above-recited sequence where X = methionine, Y = glutamine and Z =

35 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-GLU-  
 127 128 129 130 131 132 133 134 135  
 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN,  
 40 136 137 138 139 140 141 142 143

45 or the Gln<sup>137</sup> variant thereof; or X is hydrogen, Y is either a glutamine or a pyroglutamate residue and Z consists of n amino acids in the sequence 127-143 depicted in Fig. 1 hereof where n is zero or an integer of from 1 to 16, or a modification or allelic variation of the said amino acid sequence which (modification or variation) exhibits biological activity equivalent to or greater than that of gamma interferon of the amino acid sequence 1-143 depicted in Fig. 1 hereof, but excluding those of the said modifications and variations which contain any gamma interferon amino acid sequence contiguously upstream of Y or contiguously downstream of Z.

50 2. A process according to claim 1 wherein X is methionine.  
 3. A process according to claim 2 wherein Z is part or all of the sequence:

55 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
 127 128 129 130 131 132 133 134 135  
 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
 136 137 138 139 140 141 142 143

starting with GLY 127.

4. A process according to claim 1, wherein Z comprises the sequence:

5

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG  
127 128 129 130 131 132 133 134 135 136 137 138 139

10 5. A process according to Claim 1, wherein Z comprises the sequence:

GLY-LYS-ARG  
127 128 129

15

6. A process of Claim 1, wherein Z comprises the sequence:

20

GLY-LYS-ARG-LYS-ARG  
127 128 129\* 130 131

7. A process in which, following the procedure of any one of claims 1 to 6, the polypeptide is used to prepare a pharmaceutical composition.

25 8. A process according to claim 7 wherein the composition comprises a mixture of polypeptides of claim 1, in one of which Z is the sequence Gly 127 to Glu 143 and in another of which Z is the sequence Gly 127 to Arg 139, said two sequences being as set forth above.

30 9. A process according to claim 8 wherein the polypeptide in which Z is Gly 127 to Arg 139 is in a major amount relative to the polypeptide in which Z is Gly 127 to Glu 143.

10. A process according to claim 8 wherein the polypeptide in which Z is Gly 127 to Arg 139 comprises greater than about 95% of the polypeptide mixture.

35 11. A process according to claim 8 wherein the polypeptide in which Z is Gly 127 to Arg 139 comprises greater than about 97% of the polypeptide mixture.

12. A process according to any one of claims 8 to 11 wherein X is methionine.

40 13. A process according to any one of claims 7 to 12 wherein the composition comprises also a pharmaceutically acceptable level of human serum albumin.

14. A process which comprises expressing in E.coli of recombinant DNA encoding the amino acid sequence 1 to 143 as set forth above, immediately preceded by an ATG translational start codon and followed by a translational stop codon, to produce a mixture of gamma interferon polypeptides comprising:

- (i) a polypeptide of claim 1 in which n is 13, and
- (ii) a polypeptide of claim 1 in which n is 17.

50 15. A process according to claim 14 wherein species (i) is in a major amount relative to species (ii).

16. A process mixture according to claim 15 wherein the composition of the two species of gamma interferon contains greater than about 95% of species (i).

55 17. A process according to claim 16 wherein said ratio is greater than about 97% of species (i).

18. A process according to any one of claims 14 to 17 wherein X is methionine.

19. A process according to any one of claims 14 to 18 wherein species (i) Z is the amino acid sequence Gly 127 to Arg 139 as set forth above.

20. A process according to any one of claims 14 to 19 wherein in species (ii) Z is the amino acid sequence Gly 127 to Gln 143 as set forth above.

21. A process according to claim 16 wherein X comprises methionine, Z in species (i.) is the amino acid sequence Gly 127 to Arg 139 as set forth above, and Z in species (ii) is the amino acid sequence Gly 127 to Gln 143 as set forth above.

10

**Patentansprüche**

15 Patentansprüche für folgende Vertragsstaaten : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1.  $\gamma$ -Interferon-Polypeptid, bestehend aus der Aminosäure-Sequenz, die sich vom N-Terminus weg erstreckt:

20           X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-TYR-PHE-  
        1    2    3    4    5    6    7    8    9   10   11   12   13   14   15  
        ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU-  
        16   17   18   19   20   21   22   23   24   25   26   27   28   29   30   *cii*  
        25    GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
        31   32   33   34   35   36   37   38   39   40   41   42   43   44   45  
        30    GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
        46   47   48   49   50   51   52   53   54   55   56   57   58   59   60  
        LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
        61   62   63   64   65   66   67   68   69   70   71   72   73   74   75  
        35    ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-LYS-ARG-ASP-  
        76   77   78   79   80   81   82   83   84   85   86   87   88   89   90  
        ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
        91   92   93   94   95   96   97   98   99   100   101   102   103   104   105  
        40    GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
        106   107   108   109   110   111   112   113   114   115   116   117   118   119   120  
        SER-PRO-ALA-ALA-LYS-THR-  
        45    121   122   123   124   125   126

worin:

X ein Methionin-Rest ist, Y ein Glutamin-Rest ist und Z aus n Aminosäuren in der in Fig. 1 dargestellten Sequenz 127-143 besteht, worin n = 0 oder eine ganze Zahl von 1 bis 17 ist; allerdings unter Ausnahme des Lys<sup>78</sup>-Varianten-

50 Polypeptids mit der oben angegebenen Sequenz, worin X = Methionin, Y = Glutamin und Z = Gly(127)-Lys(128)-Arg(129)-Lys(130)-Arg(131)-Ser(132)-Cln(133)-Met(134)-Leu(135)-Phe(136)-Arg(137)-Gly-(138)-Arg(139)-Arg

(140)-Ala(141)-Ser(142)-Gln(143) ist, oder der Gla<sup>137</sup>-Variante davon als isoliertes Polypeptid; oder X Wasserstoff ist, Y entweder ein Glutamin- oder ein Pyroglutamat-Rest ist und Z aus n Aminosäuren in der in Fig. 1 dargestellten

55 Sequenz 127-143 besteht, worin n = 0 oder eine ganze Zahl von 1 bis 16 ist; oder eine Modifikation oder Allel-Variation der Aminosäuresequenz, die (Modifikation oder Variation) biologische Aktivität aufweist, die gleich oder höher als jene von  $\gamma$ -Interferon der in Fig. 1 dargestellten Aminosäure-Sequenz 1-143 ist, jedoch unter Ausschluß

jener Modifikationen und Variationen, die stromauf von Y oder stromab von Z eine beliebige zusammenhängende  $\gamma$ -Interferon-Aminosäure-Sequenz enthalten.

2. Polypeptid nach Anspruch 1, worin X Methionin ist.
3. Polypeptid nach Anspruch 2, worin Z ein Teil oder die Gesamtheit der Sequenz:

5

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
127 128 129 130 131 132 133 134 135

10

PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
136 137 138 139 140 141 142 143

beginnend mit GLY 127 ist.

15 4. Polypeptid nach Anspruch 1, worin Z die Sequenz:

20

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG  
127 128 129 130 131 132 133 134 135 136 137 138 139

umfaßt.

25 5. Polypeptid nach Anspruch 1, worin Z die Sequenz:

25

GLY-LYS-ARG  
127 128 129

30

umfaßt.

35 6. Polypeptid nach Anspruch 1, worin Z die Sequenz:

35

GLY-LYS-ARG-LYS-ARG  
127 128 129 130 131

40 umfaßt.

7. Replizierbares Expressionsvehikel, das in einem transformierten Mikroorganismus oder einer transformierten Zellkultur fähig ist, ein rekombinantes  $\gamma$ -Interferon-Polypeptid nach einem der Ansprüche 1 bis 6 zu exprimieren.

45 8. Expressionsvehikel nach Anspruch 7 zur Verwendung in einem Bakterium.

9. Mikroorganismus oder Zelle, der bzw. die mit dem replizierbaren Expressionsvehikel von Anspruch 7 oder 8 transformiert wurde.

50 10. Verfahren, umfassend das Kultivieren eines transformierten Mikroorganismus oder einer transformierten Zellkultur nach Anspruch 9, um das  $\gamma$ -Interferon-Polypeptid zu erzeugen.

11. Zusammensetzung, die sich zur pharmazeutischen Verabreichung eignet, umfassend ein  $\gamma$ -Interferon-Polypeptid nach einem der Ansprüche 1 bis 6.

55

12. Zusammensetzung nach Anspruch 11, umfassend ein Gemisch aus Polypeptiden nach Anspruch 1, wobei in einem davon Z die Sequenz Gly 127 bis Gln 143 und in einem anderen Z die Sequenz Gly 127 bis Arg 139 ist und beide Sequenzen wie oben definiert sind.

13. Zusammensetzung nach Anspruch 12, worin das Polypeptid, worin Z Gly 127 bis Arg 139 ist, in bezug auf das Polypeptid, worin Z Gly 127 bis Gln 143 ist, in einer größeren Menge vorliegt.
14. Zusammensetzung nach Anspruch 12, worin das Polypeptid, worin Z Gly 127 bis Arg 139 ist, mehr als etwa 95% des Polypeptid-Gemischs ausmacht.
15. Zusammensetzung nach Anspruch 12, worin das Polypeptid, worin Z Gly 127 bis Arg 139 ist, mehr als etwa 97% des Polypeptid-Gemischs ausmacht.
16. Zusammensetzung nach einem der Ansprüche 12 bis 15, worin X Methionin ist.
17. Zusammensetzung nach einem der Ansprüche 11 bis 16, weiters umfassend eine pharmazeutisch annehmbare Menge an menschlichem Serumalbumin.
18. Gemisch aus  $\gamma$ -Interferon-Polypeptiden, umfassend: (i) ein Polypeptid nach Anspruch 1, worin n = 13 ist, und (ii) ein Polypeptid nach Anspruch 1, worin n = 17 ist.
19.  $\gamma$ -Interferon-Gemisch nach Anspruch 18, worin Spezies (i) in bezug auf Spezies (ii) in einer größeren Menge vorliegt.
20.  $\gamma$ -Interferon-Gemisch nach Anspruch 19, worin die Zusammensetzung der beiden  $\gamma$ -Interferon-Spezies mehr als etwa 95% der Spezies (i) enthält.
21.  $\gamma$ -Interferon-Gemisch nach Anspruch 20, worin der Anteil der Spezies (i) größer als etwa 97% ist.
22.  $\gamma$ -Interferon-Gemisch nach einem der Ansprüche 18 bis 21, worin X Methionin ist.
23.  $\gamma$ -Interferon-Gemisch nach einem der Ansprüche 18 bis 22, worin in Spezies (i) Z die oben definierte Aminosäure-Sequenz Gly 127 bis Arg 139 ist.
24.  $\gamma$ -Interferon-Gemisch nach einem der Ansprüche 18 bis 23, worin in Spezies (ii) Z die oben definierte Aminosäure-Sequenz Gly 127 bis Gln 143 ist.
25.  $\gamma$ -Interferon-Gemisch nach Anspruch 20, worin X Methionin umfaßt, Z in Spezies (i) die oben definierte Aminosäure-Sequenz Gly 127 bis Arg 139 ist und Z in Spezies (ii) die oben definierte Aminosäure-Sequenz Gly 127 bis Gln 143 ist.
26.  $\gamma$ -Interferon-Gemisch nach Anspruch 18, das durch in E.coli stattfindender Expression rekombinanter DNA, die für die oben definierte Aminosäure-Sequenz 1 bis 143, unmittelbar hinter einem ATG-Translations-Startcodon und vor einem Translations-Stopcodon, kodiert, gebildet werden kann.

**Patentansprüche für folgenden Vertragsstaat : AT**

- 45 1. Verfahren, umfassend das Kultivieren eines Mikroorganismus oder einer Zellkultur, der bzw. die mit einem replizierbaren Expressionsvehikel transformiert wurde, um ein  $\gamma$ -Interferon-Polypeptid zu exprimieren, das aus der Aminosäure-Sequenz besteht, die sich vom N-Terminus weg erstreckt:

50

55

X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-TYR-PHE-  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15  
 5 ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU-  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
 10 GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
 15 GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60  
 LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 20 ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-LYS-ARG-ASP-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 25 GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 SER-PRO-ALA-ALA-LYS-THR-Z  
 121 122 123 124 125 126

worin:

30 X ein Methionin-Rest ist, Y ein Glutamin-Rest ist und Z aus n Aminosäuren in der in Fig. 1 dargestellten Sequenz 127-143 besteht, worin n = 0 oder eine ganze Zahl von 1 bis 17 ist; allerdings unter Ausnahme des Lys<sup>78</sup>-Varianten-Polypeptids mit der oben angegebenen Sequenz, worin X = Methionin, Y = Glutamin und Z = Gly(127)-Lys(128)-Arg(129)-Lys(130)-Arg(131)-Ser(132)-Gln(133)-Met(134)-Leu(135)-Phe(136)-Arg(137)-Gly(138)-Arg(139)-Arg(140)-Ala(141)-Ser(142)-Gln(143) ist, oder der Gla<sup>137</sup>-Variante davon als isoliertes Polypeptid; oder X Wasserstoff ist, Y entweder ein Glutamin- oder ein Pyroglutamat-Rest ist und Z aus n Aminosäuren in der in Fig. 1 dargestellten Sequenz 127-143 besteht, worin n = 0 oder eine ganze Zahl von 1 bis 16 ist; oder eine Modifikation oder Allel-Variation der Aminosäuresequenz, die (Modifikation oder Variation) biologische Aktivität aufweist, die gleich oder höher als jene von  $\gamma$ -Interferon der in Fig. 1 dargestellten Aminosäure-Sequenz 1-143 ist, jedoch unter Ausschluß jener Modifikationen und Variationen, die stromauf von Y oder stromab von Z eine beliebige zusammenhängende  $\gamma$ -Interferon-Aminosäure-Sequenz enthalten.

2. Verfahren nach Anspruch 1, worin X Methionin ist.
3. Verfahren nach Anspruch 2, worin Z ein Teil oder die Gesamtheit der Sequenz:

45 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
 127 128 129 130 131 132 133 134 135  
 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
 136 137 138 139 140 141 142 143

beginnend mit GLY 127 ist.

- 55 4. Verfahren nach Anspruch 1, worin Z die Sequenz:

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG  
127 128 129 130 131 132 133 134 135 136 137 138 139

5

umfaßt.

5. Verfahren nach Anspruch 1, worin Z die Sequenz:

10

GLY-LYS-ARG  
127 128 129

15

umfaßt.

6. Verfahren nach Anspruch 1, worin Z die Sequenz:

20

GLY-LYS-ARG-LYS-ARG  
127 128 129 130 131

25

umfaßt.

25

7. Verfahren, worin nach der Vorgangsweise nach einem der Ansprüche 1 bis 6 das Polypeptid dazu verwendet wird, eine pharmazeutische Zusammensetzung herzustellen.

30

8. Verfahren nach Anspruch 7, worin die Zusammensetzung ein Gemisch aus Polypeptiden nach Anspruch 1 umfaßt, wobei in einem davon Z die Sequenz Gly 127 bis Gln 143 und in einem anderen Z die Sequenz Gly 127 bis Arg 139 ist und beide Sequenzen oben definiert sind.

35

9. Verfahren nach Anspruch 8, worin das Polypeptid, worin Z Gly 127 bis Arg 139 ist, in bezug auf das Polypeptid, worin Z Gly 127 bis Gln 143 ist, in größerer Menge vorliegt.

40

10. Verfahren nach Anspruch 8, worin das Polypeptid, worin Z Gly 127 bis Arg 139 ist, mehr als etwa 95% des Polypeptid-Gemisches ausmacht.

45

11. Verfahren nach Anspruch 8, worin das Polypeptid, worin Z Gly 127 bis Arg 139 ist, mehr als etwa 97% des Polypeptid-Gemisches ausmacht.

50

12. Verfahren nach einem der Ansprüche 8 bis 11, worin X Methionin ist.

55

13. Verfahren nach einem der Ansprüche 7 bis 12, worin die Zusammensetzung auch eine pharmazeutisch annehmbare Menge an menschlichem Serumalbumin umfaßt.

55

14. Verfahren, umfassend die Expression in E.coli einer rekombinanten DNA, die für die oben definierte Aminosäure-Sequenz 1 bis 143, unmittelbar hinter einem ATG-Translations-Startcodon und vor einem Translations-Stopcodon, kodiert, um ein Gemisch aus  $\gamma$ -Interferon-Polypeptiden zu erzeugen, das umfaßt:

(i) ein Polypeptid nach Anspruch 1 worin n = 13 ist, und  
(ii) ein Polypeptid nach Anspruch 1, worin n = 17 ist.

55

15. Verfahren nach Anspruch 14, worin Spezies (i) in bezug auf Spezies (ii) in einer größeren Menge vorhanden ist.

16. Verfahrensgemisch nach Anspruch 15, worin die Zusammensetzung aus den beiden  $\gamma$ -Interferon-Spezies mehr als etwa 95% der Spezies (i) enthält.

17. Verfahren nach Anspruch 16, worin der Anteil der Spezies (i) größer als etwa 97% ist.

18. Verfahren nach einem der Ansprüche 14 bis 17, worin X Methionin ist.

5 19. Verfahren nach einem der Ansprüche 14 bis 18, worin in Spezies (i) Z die oben definierte Aminosäure-Sequenz Gly 127 bis Arg 139 ist.

20. Verfahren nach einem der Ansprüche 14 bis 19, worin in Spezies (ii) Z die oben definierte Aminosäure-Sequenz Gly 127 bis Gln 143 ist.

10 21. Verfahren nach Anspruch 16, worin X Methionin umfaßt, Z in Spezies (i) die oben definierte Aminosäure-Sequenz Gly 127 bis Arg 139 ist und Z in Spezies (ii) die oben definierte Aminosäure-Sequenz Gly 127 bis Gln 143 ist.

## 15 Revendications

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

20 1. Polypeptide d'interféron  $\gamma$  constitué de la séquence d'acides aminés, s'étendant à partir de l'extrémité N-terminale :

X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-LYS-TYR-PHE-  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15  
 25 ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU-  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
 30 GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
 35 GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60  
 LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 40 ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-LYS-ARG-ASP-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 45 GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 SER-PRO-ALA-ALA-LYS-THR-Z  
 50 121 122 123 124 125 126

dans laquelle:

X est un résidu méthionine, Y est un résidu glutamine et Z consiste en n acides aminés de la séquence 127-143 décrite à la Figure 1 ci-jointe où n est égal à zéro ou à un nombre entier variant de 1 à 17, mais à l'exclusion, sous forme d'un polypeptide isolé, du variant Lys 78 ayant la séquence mentionnée ci-dessus dans laquelle X représente la méthionine, Y représente la glutamine et Z représente

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG-ARG-  
 127 128 129 130 131 132 133 134 135 136 137 138 139 140

5 ALA-SER-GLN, ou son variant GLN  
 141 142 143 137

10 ou X est un atome d'hydrogène, Y est soit une glutamine soit un résidu pyroglutamate et Z consiste en n  
 acides aminés de la séquence 127-143 décrite à la Figure 1 ci-jointe où n est égal à zéro ou à un nombre entier  
 variant de 1 à 16; ou polypeptide constitué d'une modification ou d'une variation allélique de ladite séquence  
 d'acides aminés qui (s'agissant d'une modification ou d'une variation) présente une activité biologique équivalente  
 ou supérieure à celle de l'interféron  $\gamma$  de la séquence d'acides aminés 1-143 décrite à la Figure 1 ci-jointe, mais  
 15 à l'exclusion des polypeptides correspondant auxdites modifications ou variations contenant une séquence d'aci-  
 des aminés quelconque d'interféron  $\gamma$  juxtaposée en amont de Y ou en aval de Z.

2. Polypeptide selon la revendication 1, dans lequel X est une méthionine.
3. Polypeptide selon la revendication 2, dans lequel Z correspond à une partie ou à l'intégralité de la séquence:

20  
 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
 127 128 129 130 131 132 133 134 135  
 PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
 25 136 137 138 139 140 141 142 143

à partir de GLY 127.

30 4. Polypeptide selon la revendication 1, dans lequel Z comprend la séquence:

35 GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG  
 127 128 129 130 131 132 133 134 135 136 137 138 139

40 5. Polypeptide selon la revendication 1, dans lequel Z comprend la séquence:

45 GLY-LYS-ARG  
 127 128 129

50 6. Polypeptide selon la revendication 1, dans lequel Z comprend la séquence:

GLY-LYS-ARG-LYS-ARG  
 127 128 129 130 131

55 7. Vecteur d'expression doté d'un pouvoir de réplication capable, dans une culture de cellules ou de micro-organismes transformants, d'exprimer un polypeptide d'interféron  $\gamma$  recombinant tel que défini dans l'une quelconque des revendications 1 à 6.

8. Vecteur d'expression selon la revendication 7 destiné à être utilisé dans une bactérie.

9. Micro-organisme ou cellule préalablement transformé avec le vecteur d'expression doté d'un pouvoir de réPLICATION selon la revendication 7 ou la revendication 8.

5 10. Procédé qui comprend la mise en culture d'un micro-organisme ou d'une culture cellulaire transformée selon la revendication 9 en vue de produire ledit polypeptide d'interféron  $\gamma$ .

10 11. Composition appropriée à l'administration pharmaceutique comprenant le polypeptide d'interféron  $\gamma$  selon l'une quelconque des revendications 1 à 6.

12. Composition selon la revendication 11, comprenant un mélange de polypeptides selon la revendication 1, dans l'un desquels est la séquence Gly 127 à Glu 143 et dans un autre Z est la séquence Gly 127 à Arg 139, lesdites deux séquences étant telles que définies ci-dessus.

15 13. Composition selon la revendication 12, dans laquelle le polypeptide Z est Gly 127 à Arg 139 est quantitativement prépondérant par rapport au polypeptide dont Z est Gly 127 à Glu 143.

20 14. Composition selon la revendication 12, dans laquelle le polypeptide dont Z est Gly 127 à Arg 139 constitue plus de 95% environ du mélange de polypeptides.

15 15. Composition selon la revendication 12, dans laquelle le polypeptide dont Z est Gly 127 à Arg 139 constitue plus de 97% environ du mélange de polypeptides.

25 16. Composition selon l'une quelconque des revendications 12 à 15, dans laquelle X est une méthionine.

17. Composition selon l'une quelconque des revendications 11 à 16 comprenant également un taux pharmaceutiquement acceptable de sérumalbumine humaine.

30 18. Mélange de polypeptides d'interféron  $\gamma$  comprenant: (i) un polypeptide selon la revendication 1 dans lequel  $n$  est égal à 13, et (ii) un polypeptide selon la revendication 1 dans lequel  $n$  est égal à 17.

19. Mélange d'interférons  $\gamma$  selon la revendication 18, dans lequel l'espèce (i) est quantitativement prépondérante par rapport à l'espèce (ii).

35 20. Mélange d'interférons  $\gamma$  selon la revendication 19, dans lequel la composition des deux espèces d'interférons  $\gamma$  contient plus de 95% environ d'espèce (i).

21. Mélange d'interférons  $\gamma$  selon la revendication 20, dans lequel ladite proportion de l'espèce (i) est supérieure à 97% environ.

40 22. Mélange d'interférons  $\gamma$  selon l'une quelconque des revendications 18 à 21, dans lequel X est une méthionine.

23. Mélange d'interférons  $\gamma$  selon l'une quelconque des revendications 18 à 22, dans lequel Z de l'espèce (i) est la séquence d'acides aminés Gly 127 à Arg 139 telle que définie ci-dessus.

45 24. Mélange d'interférons  $\gamma$  selon l'une quelconque des revendications 18 à 23, dans lequel Z de l'espèce (ii) est la séquence d'acides aminés Gly 127 à Gln 143 telle que définie ci-dessus.

25 25. Mélange d'interférons  $\gamma$  selon la revendication 20, dans lequel X comprend une méthionine, Z de l'espèce (i) est la séquence d'acides aminés Gly 127 à Arg 139 telle que définie ci-dessus, et Z de l'espèce (ii) est la séquence d'acides aminés Gly 127 à Gln 143 telle que définie ci-dessus.

50 26. Mélange d'interférons  $\gamma$  selon la revendication 18 tel que celui pouvant être obtenu par expression dans E. coli d'ADN recombinant codant pour la séquence d'acides aminés 1 à 143 telle que définie ci-dessus, précédée immédiatement par le codon d'initiation de traduction ATG et suivie par un codon d'arrêt de traduction.

## Revendications pour l'Etat contractant suivant : AT

1. Procédé qui comprend la mise en culture d'un micro-organisme ou d'une culture cellulaire préalablement transformée avec un vecteur d'expression doté d'un pouvoir de réplication en vue d'exprimer un polypeptide d'interféron  $\gamma$  constitué de la séquence d'acides aminés, s'étendant à partir de l'extrémité N-terminale:

X-Y-ASP-PRO-TYR-VAL-LYS-GLU-ALA-GLU-ASN-LEU-LYS-TYR-PHE-  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15  
 10 ASN-ALA-GLY-HIS-SER-ASP-VAL-ALA-ASP-ASN-GLY-THR-LEU-PHE-LEU-  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
 15 GLY-ILE-LEU-LYS-ASN-TRP-LYS-GLU-GLU-SER-ASP-ARG-LYS-ILE-MET-  
 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
 20 GLN-SER-GLN-ILE-VAL-SER-PHE-TYR-PHE-LYS-LEU-PHE-LYS-ASN-PHE-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60  
 25 LYS-ASP-ASP-GLN-SER-ILE-GLN-LYS-SER-VAL-GLU-THR-ILE-LYS-GLU-  
 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 25 ASP-MET-ASN-VAL-LYS-PHE-PHE-ASN-SER-ASN-LYS-LYS-LYS-ARG-ASP-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 25 ASP-PHE-GLU-LYS-LEU-THR-ASN-TYR-SER-VAL-THR-ASP-LEU-ASN-VAL-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 30 GLN-ARG-LYS-ALA-ILE-HIS-GLU-LEU-ILE-GLN-VAL-MET-ALA-GLU-LEU-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 SER-PRO-ALA-ALA-LYS-THR-Z  
 121 122 123 124 125 126  
 35

dans laquelle:

X est un résidu méthionine, Y est un résidu glutamine et Z consiste en  $n$  acides aminés de la séquence 127-143 décrite à la Figure 1 ci-jointe où  $n$  est égal à zéro ou à un nombre entier variant de 1 à 17, mais à l'exclusion, 40 sous forme d'un polypeptide isolé, du variant Lys 78 ayant la séquence mentionnée ci-dessus dans laquelle X représente la méthionine, Y représente la glutamine et Z représente

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-Glu-PHE-ARG-GLY-ARG-ARG-  
 45 127 128 129 130 131 132 133 134 135 136 137 138 139 140

ALA-SER-GLN, ou son variant GLN  
 141 142 143 137

50 ou X est un atome d'hydrogène, Y est soit une glutamine soit un résidu pyroglutamate et Z consiste en  $n$  acides aminés de la séquence 127-143 décrite à la Figure 1 ci-jointe où  $n$  est égal à zéro ou à un nombre entier variant de 1 à 16; ou polypeptide constitué d'une modification ou d'une variation allélique de ladite séquence d'acides aminés qui (s'agissant d'une modification ou d'une variation) présente une activité biologique équivalente ou supérieure à celle de l'interféron  $\gamma$  de la séquence d'acides aminés 1-143 décrite à la Figure 1 ci-jointe, mais à 55 l'exclusion des polypeptides correspondant auxdites modifications ou variations contenant une séquence d'acides aminés quelconque d'interféron  $\gamma$  juxtaposée en amont de Y ou en aval de Z.

2. Procédé selon la revendication 1, dans lequel X est une méthionine.
3. Procédé selon la revendication 2, dans lequel Z correspond à une partie ou à l'intégralité de la séquence:

5

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-  
127 128 129 130 131 132 133 134 135

10

PHE-ARG-GLY-ARG-ARG-ALA-SER-GLN  
136 137 138 139 140 141 142 143

à partir de GLY 127.

- 15 4. Procédé selon la revendication 1, dans lequel Z comprend la séquence:

20

GLY-LYS-ARG-LYS-ARG-SER-GLN-MET-LEU-PHE-ARG-GLY-ARG  
127 128 129 130 131 132 133 134 135 136 137 138 139

- 5: Procédé selon la revendication 1, dans lequel Z comprend la séquence:

25

GLY-LYS-ARG  
127 128 129

- 30 6. Procédé selon la revendication 1, dans lequel Z comprend la séquence:

35

GLY-LYS-ARG-LYS-ARG  
127 128 129 130 131

7. Procédé dans lequel, suite aux opérations selon l'une quelconque des revendications 1 à 6, le polypeptide est utilisé pour préparer une composition pharmaceutique.
- 40 8. Procédé selon la revendication 7, dans lequel la composition comprend un mélange de polypeptides selon la revendication 1, Z de l'un d'entre eux étant la séquence Gly 127 à Glu 143 et d'un autre la séquence Gly 127 à Arg 139, lesdites deux séquences étant telles que définies ci-dessus.
9. Procédé selon la revendication 8, dans lequel le polypeptide dont Z est Gly 127 à Arg 139 est quantitativement prépondérant par rapport au polypeptide dont Z est Gly 127 à Glu 143.
- 45 10. Procédé selon la revendication 8, dans lequel le polypeptide dont Z est Gly 127 à Arg 139 constitue plus de 95% environ du mélange de polypeptides.
11. Procédé selon la revendication 8, dans lequel le polypeptide dont Z est Gly 127 à Arg 139 constitue plus de 97% environ du mélange de polypeptides.
- 50 12. Procédé selon l'une quelconque des revendications 8 à 11, dans lequel X est une méthionine.
13. Procédé selon l'une quelconque des revendications 7 à 12, dans lequel la composition comprend également un taux pharmaceutiquement acceptable de sérumalbumine humaine.
- 55 14. Procédé qui comprend l'expression dans E. coli d'ADN recombinant codant pour la séquence d'acides aminés 1

à 143 telle que définie ci-dessus, précédée immédiatement par le codon d'initiation de la traduction ATG et suivie par un codon d'arrêt de la traduction, en vue de produire un mélange de polypeptides d'interférons y compris:

5            (i) un polypeptide selon la revendication 1 dans lequel n est égal à 13, et  
              (ii) un polypeptide selon la revendication 1 dans lequel n est égal à 17.

10          15. Procédé selon la revendication 14, dans lequel l'espèce (i) est quantitativement prépondérante par rapport à l'espèce (ii).

15          16. Procédé de mélange selon la revendication 15, dans lequel la composition à base des deux espèces d'interféron gamma contient plus de 95% environ de l'espèce (i).

20          17. Procédé selon la revendication 16, dans lequel ladite proportion de l'espèce (i) est supérieure à 97% environ.

25          18. Procédé selon l'une quelconque des revendications 14 à 17, dans lequel X est une méthionine.

              19. Procédé selon l'une quelconque des revendications 14 à 18, dans lequel Z de l'espèce (i) est la séquence d'acides aminés Gly 127 à Arg 139 telle que définie ci-dessus.

              20. Procédé selon l'une quelconque des revendications 14 à 19, dans lequel Z de l'espèce (ii) est la séquence d'acides aminés Gly 127 à Gln 143 telle que définie ci-dessus.

              25. Procédé selon la revendication 16, dans lequel X comprend une méthionine, Z de l'espèce (i) est la séquence d'acides aminés Gly 127 à Arg 139 telle que définie ci-dessus, et Z de l'espèce (ii) est la séquence d'acides aminés Gly 127 à Gln 143 telle que définie ci-dessus.

30

35

40

45

50

55

## Fig. 1.

TCAAGATCAGCTATTAGAAGAGAAAGATCAGTTAAGTCCTTGGACCTGATCAGCTTGATAACAAG

30

met lys tyr thr ser  
AACTACTGATTCAACTCTTGGCTTAATTCTCTCGGAAACG ATG AAA TAT ACA AGT  
160

s10

tyr ile leu ala phe gln leu cys ile val leu gly ser leu  
TAT ATC TTG GCT TTT CAG CTC TGC ATC GTT TTG GGT TCT CTT  
150

s23 1

10

gly cys tyr cys GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS  
GGC TGT TAC TGC CAG GAC CCA TAT GTA AAA GAA GCA AAC CTT AAG

20

LYS TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY  
AAA TAT TTT AAT GCA GGT CAT TCA GAT GTA GCG GAT AAT GGA  
250

30

40

THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG  
ACT CTT TTC TTA GGC ATT TTG AAG AAT TGG AAA GAG GAG AGT GAC AGA  
300

50

LYS ILE MET GLN SER GLN ILE VAL SER PHE TYR PHE LYS LEU  
AAA ATA ATG CAG AGC CAA ATT GTC TCC TTT TAC TTC AAA CTT

60

70

PHE LYS ASN PHE LYS ASP ASP GLN SER ILE GLN LYS SER VAL GLU THR  
TTT AAA AAC TTT AAA GAT GAC CAG AGC ATC CAA AAG AGT GTG GAG ACC  
350

400

80

ILE LYS GLU ASP MET ASN VAL LYS PHE PHE ASN SER ASN LYS  
ATC AAG GAA GAC ATG AAT GTC AAG TTT TTC AAT AGC AAC AAA

90

100

LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR SER VAL THR ASP  
AAG AAA CGA GAT GAC TTC GAA AAG CTG ACT AAT TAT TCG GTA ACT GAC  
450

500

110

LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN VAL  
TTG AAT GTC CAA CGC AAA GCA ATA CAT GAA CTC ATC CAA GTG

Fig. 1. (Cont.)

120 130  
MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER  
ATG GCT GAA CTG TCG CCA GCA GCT AAA ACA GGG AAG CGA AAA AGG AGT  
550

140 143 STOP  
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN  
CAG ATG CTG TTT CGA GGT CGA AGA GCA TCC CAG TAA TGGTTGT  
600

CCTGCCTGAAATTTGAATTTAAATCTAAATCTATTATTAATTTAACATTATTTATATG  
650

GGGAATATTTTTAGACTCTCATCAATCAAATAAGTATTTATAATAGCAACTT  
700

TGTGTAATGAAATGAATATCTTAAATATGTATTATTTATAATTCCCTATATCCTGTGACTG  
750

TCTCACTTAATCCTTGTGACTAATTAGGCAAGGCTATGTGATTACAAGG  
600 850

CTTTATCTCAGGGGCCAACTAGGCAGCCAACCTAAGCAAGATCCCATGGTTGTGTTATTTC  
900

ACTTGATGATACTAACACTTATAAGTGAAGTGATACTATCCAGTTACTGCCG  
950

GTTTGAAAAATATGCCCTGCAATCTGAGCCAGTGTCTTAATGGCATGTCAGACAGAACTTGAATGT  
1000

**GTCAGGTGACCCCTGATGAAAACATAGCATCTCAGGAGATTCTGCCCTGGTGCTT**  
1050

CCAAATATTGTTGACAACTGTGACTGTACCCAAATGGAAAGTAACTCATTGTTAAATTATCA  
1100 1150

ATATCTAATATATATGAATAAGTGTAAAGTCACAACTAAAAAAAAAAAAAAAA  
1200

